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Mammalian Reovirus L3 Gene Sequences and Evidence for a Distinct Amino-Terminal Region of the λ1 Protein

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To complement evidence for nucleoside triphosphate phosphohydrolase (NTPase), RNA helicase, RNA 5′ triphosphate phosphohydrolase, and nucleic acid-binding activities by the core shell protein λ1 of mammalian orthoreoviruses (reoviruses), we determined nucleotide sequences of the λ1-encoding L3 gene segments from three isolates: type 1 Lang (T1L), type 2 Jones (T2J), and type 3 Dearing (T3D). The T1L and T3D L3 gene sequences and deduced λ1 protein sequences shared high levels of identity (97.7% and 99.3%, respectively). The λ1 sequences differed at only 9 of 1275 amino acids. Two single-nucleotide insertions relative to a previously published sequence for T3D L3 extended the λ1 open reading frame to within 60 nucleotides of the plus-strand 3′ end for T3D and the other isolates sequenced, in keeping with the short 3′ nontranslated regions of the other nine gene segments. Seven of the nine amino acid differences between T1L and T3D λ1 were located within the amino-terminal 500 residues of λ1, a region with putative sequence similarities to NTPases and RNA helicases. The T2J L3 and λ1 sequences were found to be more divergent, especially within the amino-terminal 180 residues of λ1, preceding the putative CCHH zinc finger motif. The T2J L3 sequence, along with partial sequences for L3 genes from three other reovirus isolates, suggested that one or more of the polymorphisms at amino acids 71, 215, 500, 1011, and/or 1100 in λ1 contribute to the L3-determined differences in ATPase activities by T1L and T3D cores. The findings contribute to our ongoing efforts to elucidate sequence-structure–function relationships for the λ1 core protein.

INTRODUCTION

We recently reported that the L3 gene is a genetic determinant of strain differences in ATPase activities by core particles of mammalian orthoreoviruses (reoviruses) type 1 Lang (T1L) and type 3 Dearing (T3D) (Noble and Nibert, 1997a). L3 was identified as the primary determinant in two different genetic analyses: one involving the rates of ATP hydrolysis by T1L and T3D cores at 35°C, pH 8.5, with low monovalent ion concentrations, and the other involving the relative rates of ATP hydrolysis by cores under similar conditions with or without 150 mM potassium acetate. L3 encodes the λ1 protein, one of two major proteins in the inner (core) capsid in reovirus particles. Although the genetic mappings do not prove that λ1 is an ATPase, we suggested that it may hydrolyze ATP as part of an RNA helicase activity in cores (Noble and Nibert, 1997a). This speculation was based on putative sequence similarities between the N-terminal region of λ1, as deduced from the published sequence of T3D L3 (Bartlett and Joklik, 1988) and RNA helicases (Gorbalenya and Koonin, 1993).

Subsequent findings by Bisaillon et al. (1997) and Bisaillon and Lemay (1997a) provided direct evidence that the λ1 protein expressed in *Pichia pastoris* has both nucleoside triphosphate phosphohydrolase (NTPase) and RNA helicase activities, as well as an RNA triphosphatase activity that may be involved in mRNA capping. In addition, Bisaillon *et al.* (1997) identified other putative helicase motifs within the N-terminal 440 amino acids of λ1. Although apparently robust, these results leave open to some question which function or functions λ1 truly executes during RNA synthesis and mRNA capping by reovirus. They also do not address whether other protein cofactors may contribute to these activities. For example, another recent study from our laboratory identified core protein μ2 as a second genetic determinant of differences in NTP hydrolysis by reovirus T1L and T3D cores (Noble and Nibert, 1997b). This suggests that μ2 is either a second NTPase in cores or serves to modulate the activity of λ1.

Other results have demonstrated the capacity of λ1 to bind both double-stranded RNA (Schiff *et al.,* 1988; Lemay and Danis, 1994; Bisaillon and Lemay, 1997b) and single-stranded RNA (Bisaillon and Lemay, 1997b) but with a greater affinity for the latter (Bisaillon and Lemay, 1997b). Sequences near the N-terminus of λ1 are essential for this activity (Lemay and Danis, 1994; Bisaillon and

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Lemay, 1997b), but a putative CCHH zinc finger motif at amino acids 183–206 in T3D λ1 (Bartlett and Joklik, 1988; Schiff *et al.,* 1988; Dermody *et al.,* 1991; Lemay and Danis, 1994) is apparently not essential for RNA binding (Lemay and Danis, 1994; Bisaillon and Lemay, 1997b). RNA-binding acitivity by λ1 is consistent with its putative roles in RNA helicase and RNA 5′ triphosphate phosphohydrolase activities.

New evidence for the locations of λ1 and μ2 in reovirus particles has also come from recent work. Dryden *et al.* (1998) used cryoelectron microscopy and three-dimensional image reconstruction to identify inwardly projecting structures in reovirus top component particles, which lack the genomic double-stranded RNA that obscured the particle interior in earlier studies (Metcalf *et al.,* 1991; Dryden *et al.,* 1993). The internal structures are centered at the particle fivefold axes and appear to be formed from portions of proteins λ1 and μ2, as well as from the reovirus RNA polymerase protein λ3. In particular for λ1, an N-terminal 37-kDa region containing all but the most C-terminal of the putative NTPase and helicase sequence motifs (Noble and Nibert, 1997a; Bisaillon *et al.,* 1997), was identified by proteolysis and protein sequencing as a region of λ1 that is likely to extend beneath the core shell (Dryden *et al.,* 1998). A study by Coombs (1998) confirmed that λ1 is present in 120 copies per particle (Smith *et al.,* 1969) and indicated that μ2 is present in ~20 copies per particle, suggesting that a dimer of μ2 may be present at or near each particle fivefold axis. Important remaining questions about reovirus structure include how the 120 copies of λ1 and the other major core protein σ2 are arranged within the core shell.

For the current study, to address whether specific residues in λ1 segregate with the L3-determined differences in ATP hydrolysis by reovirus T1L and T3D cores (Noble and Nibert, 1997a), we sequenced the T1L L3 gene for comparison with the previously reported sequence for T3D L3 (Bartlett and Joklik, 1988). The N-terminal 440 residues of T1L λ1 were of greatest interest due to the putative sequence similarities with other NTPases within that region of T3D λ1 (Bartlett and Joklik, 1988; Noble and Nibert, 1997a; Bisaillon *et al.,* 1997). We also noted that the 3′ nontranslated region of the previously published T3D L3 sequence (Bartlett and Joklik, 1988) is much longer than that reported for all other reovirus gene segments. Moreover, computer-generated translations of the previous sequence in its three reading frames indicated that a single-nucleotide insertion between nucleotides 3625 and 3714 would extend the open reading frame for λ1 to much nearer the 3′ end of the L3 plus strand (data not shown). This change would cause the length of the 3′ nontranslated region to be only 35 nucleotides (versus 181 nucleotides in the previous sequence), putting it within the range of the other nine T3D gene segments (32–80 nucleotides) (reviewed in Dermody *et al.,* 1991). We therefore also sequenced the T3D L3 gene to test whether the previous sequence is missing at least one nucleotide in the 3625–3714 region. Last, to gain further insight into sequence determinants of the L3-determined differences in ATP hydrolysis among reovirus isolates, as well as to address whether other isolates may exhibit greater sequence variation in their λ1 proteins than we observed for T1L and T3D, we sequenced the L3 gene of reovirus type 2 Jones (T2J) and portions of L3 from three other isolates. By examining λ1 sequence variation and correlating it with differences in enzymatic activity, the work described in this report contributes to our ongoing efforts to elucidate sequence–structure–function relationships among the reovirus core proteins.

TABLE 1

Identities in Pairwise Alignments of L3 Nucleotide and Deduced λ1 Amino Acid Sequences

	Percent identity in pairwise comparisons between virus strains		
Gene or protein	T1L:T2J	T2J:T3D	T1L:T3D
L3	76.5	76.5	97.7
λ1	95.6	95.5	99.3

RESULTS AND DISCUSSION

Comparison of nucleotide sequences of T1L and T3D L3 genes

Sequences of the T1L and T3D L3 genes (GenBank accession numbers AF129820 and AF129822, respectively) were found to be 3901 nucleotides long, to contain no insertions or deletions relative to one another when aligned, and to be identical at 3811 positions (97.7% of total) (Table 1). This makes L3 the second most conserved of sequenced gene segments from these two virus isolates, with only the M1 genes of T1L and T3D being slightly more conserved (97.8%) according to previously reported sequences (Wiener *et al.,* 1989; Zou and Brown, 1992).

There are 90 nucleotide differences between the new T1L and T3D L3 gene sequences. Eight of the 13 differences at first positions in codons and 71 of the 72 differences at third positions in codons are silent in the deduced λ1 protein sequence (Table 2). These values reflect the high degree of conservation between the L3 genes and λ1 proteins of these two isolates. In addition, the low frequency of L3 nucleotide changes suggest that the two L3 genes only recently diverged or that there are selective pressures to maintain sequences at the RNA level. No clustering of nucleotide differences between the T1L and T3D L3 genes is readily apparent. In addition to differences in the coding regions, we found differ-

TABLE 2

Positions of Differences in Pairwise Comparisons of L3 Nucleotide Sequences

Codon position	Differences in pairwise comparisons between virus strains		
	T1L:T2J	T2J:T3D	T1L:T3D
1	117	121	13
2	32	31	3
3	759	756	72
5′ NT[a]	0	0	0
3′ NT[a]	7	9	2
Total	915	917	90

[a] 5′ and 3′ nontranslated regions.

ences at two positions, 3872 and 3879, in the 3′ nontranslated regions of the T1L and T3D L3 genes (Table 2, Fig. 1).

The identified nucleotide differences between T1L and T3D L3 accurately reflect seven restriction site polymorphisms in DNA copies of the T1L and T3D L3 genes that were previously identified by empirical means and used to evaluate the origin of the L3 gene segments in 84 T1L × T3D reassortant viruses (Nibert *et al.,* 1996a; Margraf and Nibert, unpublished data). In addition, sites for cleavage by five other restriction enzymes that were noted to be shared by T1L and T3D L3 in the previous study were also reflected in the sequences.

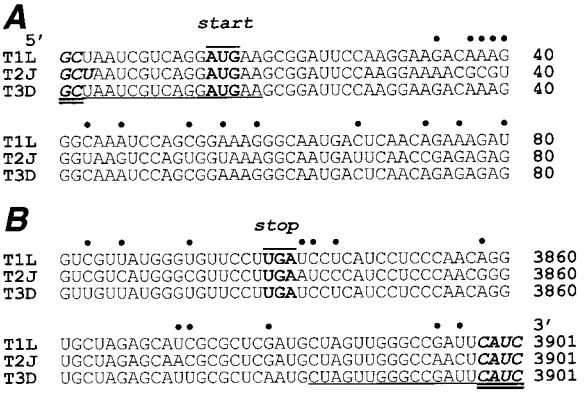

FIG. 1. Sequences near the 5′ (A) and 3′ (B) ends of the reovirus T1L, T2J, and T3D L3 genes, plus strands. The start and stop codons for translation of λ1 are indicated in bold. Positions at which at least one sequence differs from the others are indicated by filled circles. The 18 nucleotides at each end that were fixed by primers in the initial amplification protocol are underlined. The two or four nucleotides at each end that were fixed by primers in the RACE protocols are double underlined. The displayed sequences are those obtained after RACE, which were the same as those obtained after the initial protocol except for the two differences nearest the 3′ end of T2J L3, which were fixed by the 3′ primer in the initial protocol. The two to four nucleotides at each end that were fixed by primers or were otherwise not readable in the sequences obtained after RACE are indicated by bold italics.

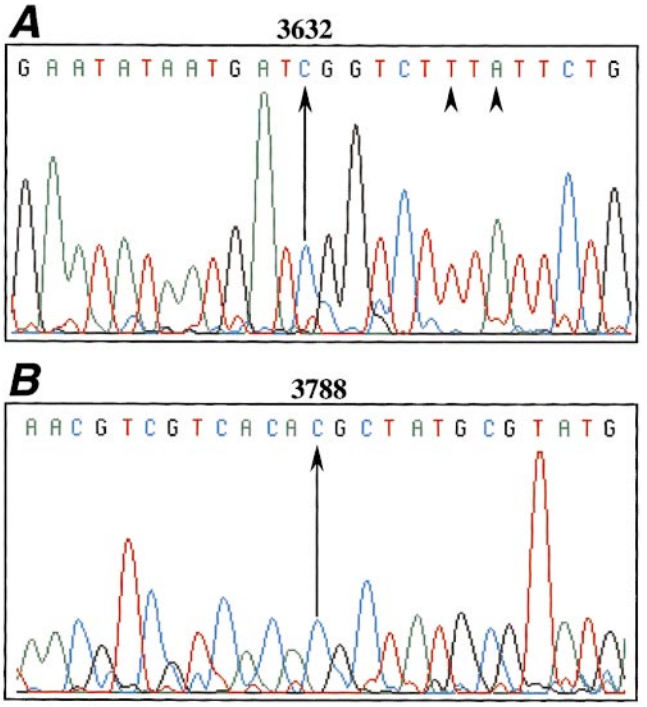

FIG. 2. Electropherograms from automated DNA sequence analysis of the L3 gene from reovirus T1L, showing sequences surrounding nucleotide 3632 (A) and 3788 (B). For both panels, sequences representing the L3 plus strand are shown and were generated using a primer with 3′ end corresponding to L3 nucleotide 3480. Nucleotides 3632 and 3788 (arrows) represent single-base insertions relative to the previously published sequence for the T3D L3 gene (Bartlett and Joklik, 1988). Sequence differences between our T1L and T3D sequences in the illustrated regions are highlighted by arrowheads. Sequences were generated from noncloned PCR products of the T1L L3 gene as described in Materials and Methods, and the data were displayed using the computer program EditView 1.0.1 (Perkin–Elmer).

Nucleotide insertions relative to the previously published T3D L3 sequence

Especially notable in the newly determined T1L and T3D L3 sequences is that they each contain five insertions relative to the T3D L3 sequence of Bartlett and Joklik (1988). There are three single-base insertions near position 1500 (positions 1497, 1503, and 1505 in our sequences) and two single-base insertions nearer the 3′ end of L3 (positions 3632 and 3788) (Fig. 2). The insertions near position 1500 constitute one extra codon and thus do not affect the frame in which the more C-terminal regions of λ1 are translated. The two insertions near the 3′ end, however, result in two consecutive frameshifts that extend the open reading frame for λ1 to much nearer the 3′ end of L3 than in the previous sequence (Bartlett and Joklik, 1988) (Fig. 1). The fact that all five of these insertions were found at the same positions and with the same inserted bases in both T1L and T3D sequences obtained from both plasmid clones and noncloned PCR products argues for their validity. In addition, we performed restriction enzyme digestions on a DNA product

from a portion of the T3D L3 gene because the insertion at 3632 in our sequence creates an additional site for *Sau*3AI and the insertion at 3788 in our sequence abolishes a site for *Alu*I, relative to the sites predicted by the previously published sequence. With each enzyme, the restriction pattern we observed was that predicted by our own T3D L3 sequence (data not shown).

As a consequence of the longer open reading frame for λ1, the 3′ nontranslated region of L3 is shortened from the 181 nucleotides reported previously (Bartlett and Joklik, 1988). The 3′ nontranslated regions of both T1L and T3D L3 genes consist of 60 nucleotides (differing from the 38 nucleotides predicted above because of the additional insertion at nucleotide 3788) (Fig. 1). The 3′ nontranslated regions of all 10 gene segments of reovirus T3D thus have lengths between 32 and 80 nucleotides, with the longest four being those of M1 (80), S3 (70), S4 (66), and L3 (60) (reviewed in Dermody *et al.,* 1991). The 5′ nontranslated regions of the 10 T3D gene segments are also small (12–32 nucleotides; 13 for L3) (Fig. 1), reflecting the economy of the reovirus genome with regard to sequences outside the protein-coding regions that may be required for RNA packaging or replication.

In addition to the inserted nucleotides, the T3D L3 sequence reported here includes substitutions at nine nucleotide positions relative to the previously published sequence (Bartlett and Joklik, 1988). Of these nine substitutions, seven result in differences in the λ1 amino acid sequences deduced from the new and old T3D L3 sequences (at positions 119, 174, 500, 583, 648, 735, and 852 in the new sequence). For eight of the nucleotide substitutions and six of the amino acid differences, however, the newly deduced T1L and T3D L3 and λ1 sequences are conserved at those positions (Fig. 3), suggesting that most of the nucleotides unique to the old T3D sequence may represent sequencing errors.

Evidence that λ1 proteins expressed from cloned L3 genes are properly terminated

To address whether the cloned T1L and T3D L3 genes encode a λ1 protein of the correct length, we analyzed the proteins by *in vitro* transcription and translation (see Materials and Methods). In initial experiments, the mobility of *in vitro* translated [^{35}S]methionine/cysteine-labeled T1L λ1 protein obtained from the cloned T1L L3 gene was compared with that of λ1 protein from [^{35}S]methionine/cysteine-labeled T1L virions using either reduced-bis Tris–glycine SDS gels (see Materials and Methods), in which λ1 and λ2 migrate similarly but are resolved from λ3 (Cleveland *et al.,* 1986; Nibert *et al.,* 1996a), or phosphate–urea SDS gels, in which λ1 exhibits a distinct mobility from either λ2 or λ3 (Smith *et al.,* 1969). Both types of gels showed comigration of λ1 from the two sources (Figs. 4A and 4B), consistent with the λ1 protein derived from the cloned T1L L3 gene being the same length as λ1 from T1L virions.

To obtain further evidence for a longer λ1 open reading frame than was previously reported, we used site-directed mutagenesis to delete nucleotide 3632 (numbering in our sequences) from our T3D L3 clone. This mutation resulted in a clone with the sequence reported by Bartlett and Joklik (1988) in that region and caused translation of λ1 to terminate after nucleotide 3716 (numbering in our sequences) as reported by them, rather than after nucleotide 3838, as in our wild-type T1L and T3D L3 clones. The wild-type and mutated T3D L3 clones were then used for *in vitro* transcription and translation, and the translation products were compared with the λ1 protein from T3D virions by electrophoresis in reduced-bis gels. The *in vitro* translated, mutated λ1 protein was found to migrate distinctly faster than the virion-derived λ1 in this case (Fig. 4C), demonstrating that termination of λ1 translation normally occurs at a site more 3′ in the L3 transcript than that predicted by Bartlett and Joklik (1988). In contrast, the λ1 protein translated from our original plasmid comigrated with virion-derived λ1 (Fig. 4C), providing evidence that our cloned T3D L3 gene encodes a properly terminated λ1 protein.

Determination of nucleotide sequence of reovirus T2J L3 gene

To gain further information on both L3 and λ1 sequences and to better identify functionally important regions in the λ1 protein, we determined the nucleotide sequence of the L3 gene of reovirus isolate T2J (GenBank accession number AF129821). The T2J L3 gene was found to have the same overall length (3901 nucleotides) and to possess nontranslated regions of the same sizes (13 and 60 bases for the 5′ and 3′ ends, respectively) as the T1L and T3D L3 genes (Fig. 1; data not shown). Moreover, the T2J L3 gene was found to contain the same five insertions relative to the published T3D L3 sequence (Bartlett and Joklik, 1988) as were found in our T1L and T3D L3 sequences (see above). Overall, however, the T2J L3 gene was much more highly divergent from the T1L and T3D L3 genes (76.5% identity for each at the nucleotide level) than the latter are from each other (Table 1). T2J L3 contains 915 nucleotide changes from T1L L3 and 917 nucleotide changes from T3D L3, as described further in Table 2. The changes resulting in amino acid differences cluster toward the 5′ end of T2J L3 (see below). The finding of greater sequence divergence in the T2J L3 gene argues against an hypothesis that the extreme conservation of nucleotide sequences in the T1L and T3D L3 genes reflects selective pressure to maintain sequences at the RNA level. The greater divergence of T2J L3 is consistent with published findings for other T2J genes (Wiener *et al.,* 1989; Dermody *et al.,* 1991; Seliger *et al.,* 1992).

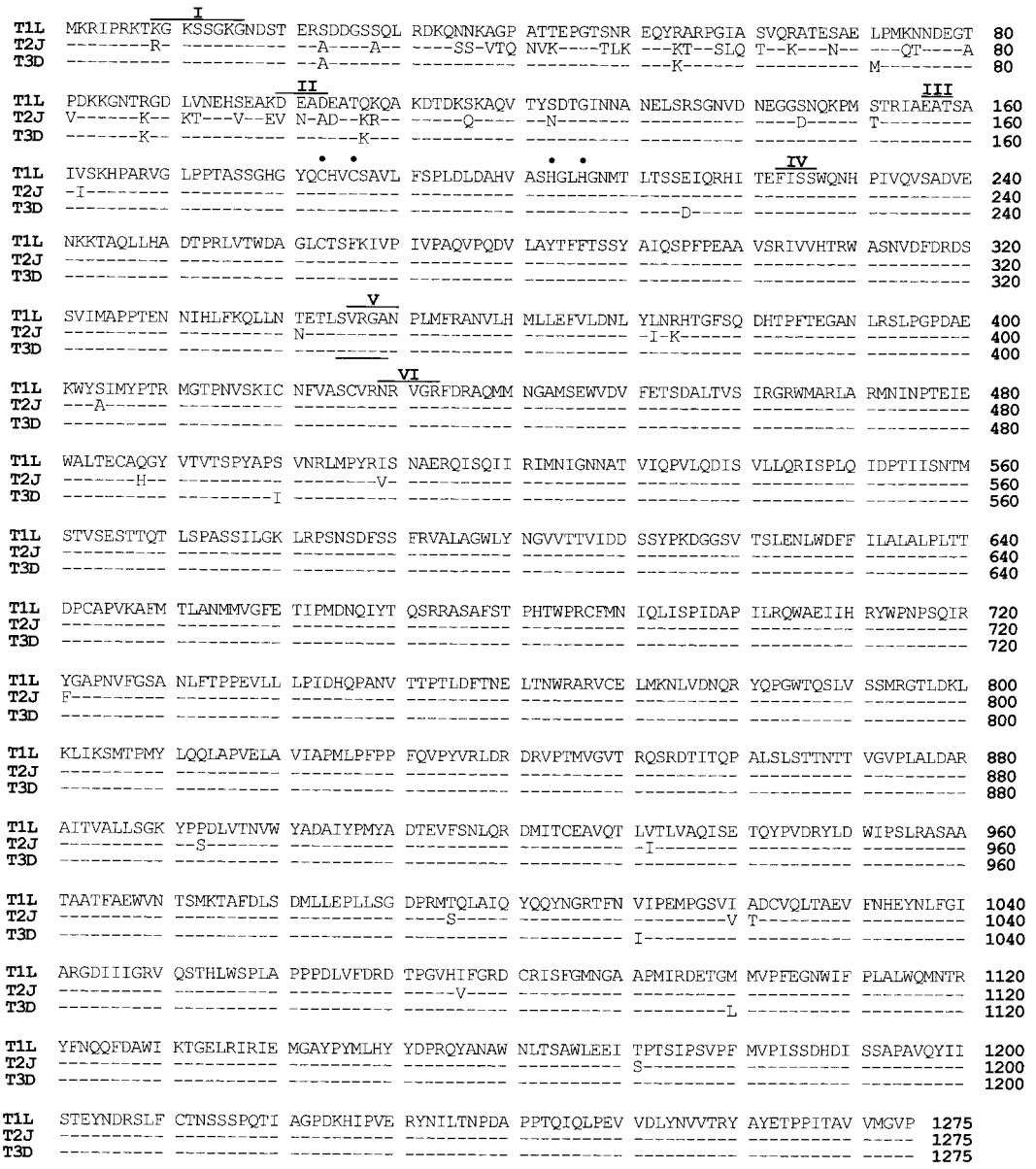

FIG. 3. Amino acid sequences for the reovirus T1L, T2J, and T3D λ1 proteins as deduced from the L3 nucleotide sequences determined in this study. Amino acids are designated by single-letter code. Amino acids shared with T1L are indicated by dashes for T2J and T3D. Sequence motifs putatively shared with RNA helicases in an N-terminal portion of λ1 (numbered I–VI; Bisaillon *et al.,* 1997) are indicated by labels and overlines. Cysteine and histidine residues proposed to mediate zinc binding by λ1 (Bartlett and Joklik, 1988; Schiff *et al.,* 1988; Dermody *et al.,* 1991; Lemay and Danis, 1994) are topped by filled circles. The N-terminal sequence determined for the C-terminal fragment of λ1 that remains resistant to chymotrypsin digestion in T3D top component particles (Dryden *et al.,* 1998) is indicated by underlining at positions 345–349.

Determination of nucleotide sequences from the ends of L3 genes

Our initial methods involved fixing the sequence of 18 nucleotides at each end of the L3 genes with the primers used in cDNA production and amplification (primers designed from the published T3D L3 sequence; Bartlett and Joklik, 1988). The fact that these primers were productive at amplifying the L3 genes of reoviruses T1L, T2J, and T3D indicated that sequences in the terminal regions are strongly conserved among these isolates. For completeness, however, we also determined sequences for the ends of L3 from DNA products in which only very small regions (two or four nucleotides) at each end of the genes had been fixed by primers. Protocols for 5′ rapid amplification of cDNA ends (RACE) and 3′ RACE (Frohman *et al.,* 1988) were used on the L3 genes of isolates T1L, T2J, and T3D for these analyses (see Materials and Methods). Within the regions fixed by primers in the preceding analyses, all sequences from the three isolates were found to agree, with the exception of two differences at positions 3894 and 3896 near the 3′ end of T2J L3 (Fig. 1). These findings agree with the sequences

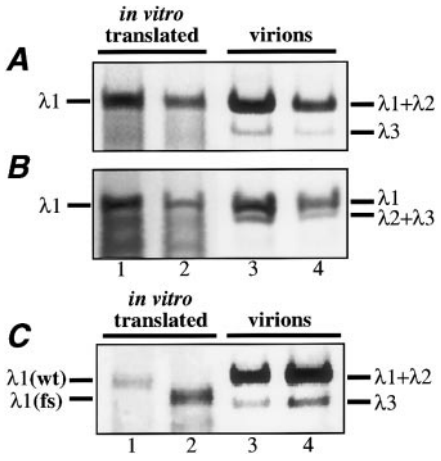

FIG. 4. Migration of λ1 protein from mammalian reovirus virions compared with that from *in vitro* translations of RNA transcribed from L3 clones. (A) Reduced-bis Tris–glycine SDS 4–9% polyacrylamide gradient gel of 15 μl (lane 1) or 10 μl (lane 2) *in vitro* translated T1L λ1 run alongside 1×10^{11} (lane 3) or 5×10^{10} (lane 4) [^{35}S]methionine/cysteine-labeled T1L virions. (B) Phosphate–urea SDS 7.5% polyacrylamide gel of 10 μl (lane 1) or 5 μl (lane 2) *in vitro* translated T1L λ1 run alongside 1×10^{11} (lane 3) or 5×10^{10} (lane 4) [^{35}S]methionine/cysteine-labeled T1L virions. (C) Reduced-bis Tris–glycine SDS 4–9% polyacrylamide gradient gel of 7.5 μl *in vitro* translated T1L λ1 (lane 1) or 7.5 μl of *in vitro* translated T1L λ1 derived from the L3 clone engineered to delete nucleotide 3632 (lane 2) run alongside 1.25×10^{10} (lane 3) or 2.5×10^{10} (lane 4) [^{35}S]methionine/cysteine-labeled T1L virions. The mobilities apparent for full-length, wild-type (wt) λ1 and truncated, frameshifted (fs) λ1 are indicated.

for the extreme terminal regions of T1L, T2J, and T3D L3 reported by Gaillard *et al.* (1982). The two changes in T2J were included in the comparisons with T1L and T3D discussed in the preceding section. The longest consecutive region of identical nucleotides among the three sequences is at positions 1–32, that is, at the extreme 5′ end of the coding strand (Fig. 1; data not shown).

Comparison of deduced amino acid sequences of T1L, T2J, and T3D λ1 proteins

The T1L, T2J, and T3D λ1 proteins are each deduced to be 1275 amino acids long and to contain no insertions or deletions relative to one another (Fig. 3). T1L and T3D λ1 proteins share identity at 1266 positions (99.3%) (Table 1), making λ1 the most conserved protein from these two isolates based on deduced amino acid sequences. The σ2 and μ2 proteins of T1L and T3D are next most conserved, exhibiting 98.8% and 98.6% deduced sequence identity, respectively, according to previously reported sequences (Wiener *et al.,* 1989; Dermody *et al.,* 1991; Zou and Brown, 1992). In contrast, T2J λ1 shares only 95.6% and 95.5% deduced sequence identity with the T1L and T3D λ1 proteins, respectively (Table 1). This makes T2J λ1 second to T2J μ1 in level of deduced sequence identity with T1L and T3D proteins according to reported sequences (97.2% and 96.8%, respectively, for μ1; Jayasuriya *et al.,* 1988; Wiener and Joklik, 1988).

Seven of the nine deduced amino acid differences between T1L and T3D λ1 are found near the N-terminus, between residues 23 and 500. Similarly, 47 of 56 differences between T1L and T2J λ1 and 46 of 57 differences between T2J and T3D λ1 are found within the N-terminal 500 amino acids of these proteins. In fact, the greatest divergence is restricted to the N-terminal 180 residues of these proteins, preceding the putative CCHH zinc finger motif conserved in each (Fig. 3). Specifically, 42 of the 56 differences between T1L and T2J λ1, 39 of the 57 differences between T2J and T3D λ1, and 5 of the 9 differences between T1L and T3D λ1 are found within the N-terminal 180 amino acids of these proteins.

The striking localization of sequence divergence to the N-terminal 150–200 amino acids of the T2J λ1 protein was further highlighted by a computer-based analysis for sequence identities along the lengths of the λ1 sequences (Fig. 5) and is interesting for several reasons. First, the N-terminal 344 amino acids of λ1 were inferred from their proteinase sensitivity in top component particles to project into the interior of reovirus particles, as opposed to the rest of the protein, which was inferred to form the core shell (Dryden *et al.,* 1998). Thus according to this model, the N-terminal region of λ1 may tolerate sequence changes more readily than the rest of the protein because it has fewer structural constraints than imposed by regular protein contacts within the shell. Second, the L3-determined differences in ATPase activities by T1L and T3D cores may be determined by one or more of the seven deduced differences within this N-terminal region because this region contains the putative NTPase and helicase motifs. These amino acid differences (Fig. 3) include four (residues 23, 54, 71, and 88) between putative helicase motifs I and II (Bartlett and Joklik, 1988; Noble and Nibert, 1997a; Bisaillon *et al.,* 1997), one (residue 107) immediately after putative motif II, and one (residue 215) immediately before putative

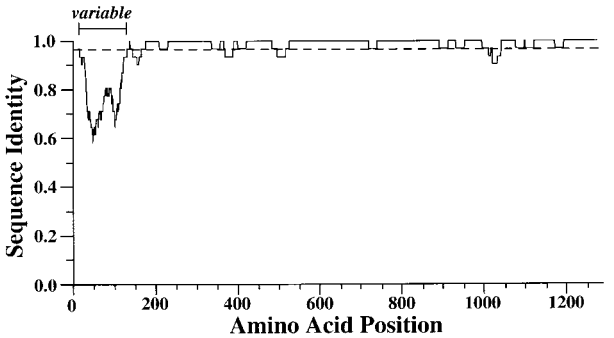

FIG. 5. Window-averaged calculations for sequence identity among the reovirus T1L, T2J, and T3D λ1 proteins. Data and graphics were generated with the program Plotsimilarity in GCG 9.1 (Genetics Computer Group). For the analysis shown, the program was set to average identities among the three λ1 proteins over running windows of 21 amino acids. An N-terminal region of variable sequences among the three virus isolates is marked. The average identity score is indicated by a dashed line.

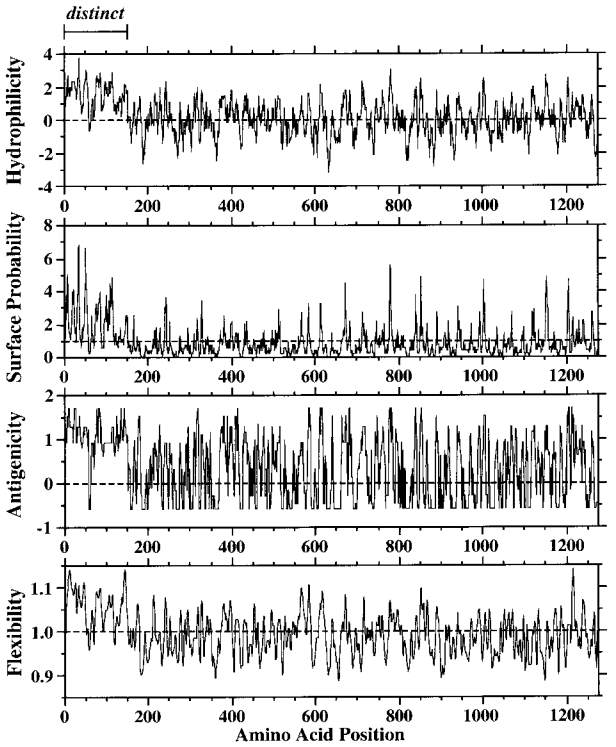

FIG. 6. Window-averaged calculations for hydrophilicity, surface probability, antigenicity, and flexibility in the reovirus T1L λ1 protein. Data were generated with the programs Peptidestructure (default settings) and Plotstructure in GCG 9.1 (Genetics Computer Group). An N-terminal region with distinct characteristics from the remainder of the protein in these analyses is marked. The average residue value for each property is indicated by a dotted line.

motif IV (Bisaillon *et al.,* 1997) and after the putative zinc finger motif (Bartlett and Joklik, 1988; Schiff *et al.,* 1988; Dermody *et al.,* 1991; Lemay and Danis, 1994) that are conserved in T1L and T3D λ1. Interestingly, helicase motif II is only poorly conserved in the T2J λ1 sequence. Specifically, the DEADE sequence assigned to motif II in T1L and T3D λ1 is replaced by VNAAD in T2J λ1 (Fig 3). The altered motif II in T2J may retain its proposed significance for helicase function by λ1 (Noble and Nibert, 1997a; Bisaillon *et al.,* 1997) or may instead indicate that the similarity of this sequence in T1L and T3D λ1 to that of motif II in helicases is fortuitous.

Other predicted features of deduced λ1 sequences

Computer-based analyses for hydrophilicity, surface probability, antigenic index, and flexibility along the lengths of the T1L, T2J, and T3D λ1 proteins provided additional evidence that the N-terminal part of λ1 has distinct characteristics. In particular, the N-terminal 150–200 amino acids in T1L (Fig. 6) and T3D (data not shown) λ1 proteins exhibited high values for these properties. Similar results were obtained for the N-terminal 150–200 amino acids of T2J λ1 (data not shown), despite its more divergent sequence within this region. These findings correlate with previous evidence that an N-terminal region of λ1 projects below the main core shell (Dryden *et al.,* 1998), exhibits nucleic acid binding activity (Lemay and Danis, 1994; Bisaillon and Lemay, 1997b), and is proposed to be involved in ATPase and other enzymatic activities by reovirus cores (Noble and Nibert, 1997a; Bisaillon *et al.,* 1997; Bisaillon and Lemay, 1997a). The greater hydrophilicity of an N-terminal portion of λ1 is also evident from the distribution of charged residues in the protein, including a high frequency of basic residues within the N-terminal 150–200 amino acids (Fig. 3), which may be important for interactions with RNA (Lemay and Danis, 1994; Bisaillon and Lemay, 1997b). Choosing amino acid 180 as an arbitrary cutoff between N- and C-terminal portions of λ1, we calculated isoelectric points of 9.88 and 5.56 for the two regions in T1L λ1 versus 6.05 for the whole protein. The respective values were calculated to be 10.15, 5.64, and 6.23 for T2J λ1 and 10.00, 5.56, and 6.12 for T3D λ1. These results provide additional evidence that an N-terminal region of λ1 has a distinct character from the rest of the protein.

The molecular weights calculated from the newly deduced λ1 sequences were 141879 for T1L, 142029 for T2J, and 141833 for T3D. These compare with 137353 calculated from the deduced T3D λ1 sequence of Bartlett and Joklik (1988). Previous data for the molecular weight and copy number of reovirus core components do not account for the full mass of these particles as determined by hydrodynamic or microscopic methods (Farrell *et al.,* 1974; reviewed in Nibert, 1998). Core mass according to both of those experimental methods is approximately 52×10^6. Previous data for λ1 (120 copies per core), other core proteins, and the dsRNA genome yielded a calculated mass of 48.6×10^6 for T3D cores (Nibert, 1998). The additional mass provided by the longer λ1 protein identified in this study and the larger copy number (20 versus 12) recently suggested for core protein μ2 (Coombs, 1998) increases the calculated mass to 49.8×10^6, but still leaves approximately 2×10^6 unaccounted for.

ATPase phenotypes of reovirus isolates and tentative sequence determinants in λ1

Noble and Nibert (1997a) reported two distinct measures of core ATPase activities that differ between reoviruses T1L and T3D and that segregate uniquely with the L3 gene among reassortants of these strains. One difference involved the rate of ATP hydrolysis at pH 8.5 and 37°C under low-salt conditions, and the other difference involved the rate of ATP hydrolysis with or without 150 mM potassium acetate. We suggested that these differences may be mechanistically linked and explained by a single amino acid difference between the T1L and T3D λ1 proteins (Noble and Nibert, 1997a). To address whether the rate of ATP hydrolysis and its response to

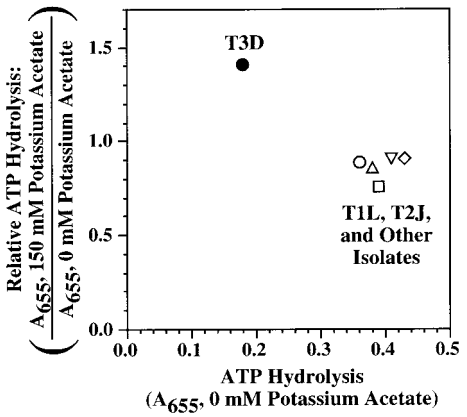

FIG. 7. ATPase activities of reoviruses T1L, T2J, T3D, and other isolates. ATPase activities for cores of reoviruses T1L (○), T2J (□), T3D (●), T3c10 (△), T3c18 (◇), and T3c87 (▽) were determined at two different sets of conditions: in the absence of potassium acetate or in the presence of 150 mM potassium acetate as defined by Noble and Nibert (1997a). Symbols denote the means of two determinations at each set of conditions for each isolate.

potassium acetate are related, we examined the ATPase phenotypes of nine additional isolates of mammalian reovirus including T2J. Cores of all these isolates behaved with striking similarity to T1L both in hydrolyzing ATP rapidly with no added salt and less rapidly in the presence of 150 mM potassium acetate (Fig. 7 and data not shown). These findings are consistent with the two L3-determined ATPase phenotypes being causally linked and explained by a single amino acid difference between the λ1 proteins of T3D and other strains. However, it is also possible that the T3D λ1 protein contains two or more unique amino acids that independently determine its two unique, L3-determined ATPase phenotypes. It must be noted that because only T1L × T3D reassortants were used to assign these differences in ATPase activities to the L3 genes of T1L and T3D, we cannot rule out the possibility that other proteins may play a role in determining these behaviors with the other isolates. However, the strong similarities of the other isolates to T1L in these two measures of ATPase activity suggest a common basis in their L3 genes.

In an effort to pinpoint which of the nine amino acid differences between T1L and T3D λ1 are responsible for the L3-determined differences in ATPase phenotypes of these isolates, we first analyzed the deduced λ1 sequence of T2J. The T2J sequence revealed amino acids identical to T3D at four of the nine positions (alanine 23, lysine 54, lysine 88, and lysine 107) and identical to T1L at the remaining five positions (leucine 71, glutamate 215, serine 500, valine 1011, and methionine 1100) (summarized in Table 3). Because T2J and T1L cores exhibit very similar ATPase phenotypes (Fig. 7), the simplest interpretation is that one or more of the amino acids they share at positions 71, 215, 500, 1011, and 1100 cause their L3-determined ATPase phenotypes to be distinct from those of T3D.

In an effort to confirm the importance of particular amino acid differences between T1L and T3D λ1 for their L3-determined differences in ATPase phenotypes, we also determined partial sequences for the L3 genes of three of the additional isolates that we tested for ATPase activity: type 3 clones 10 (T3c10), 18 (T3c18), and 87 (T3c87) (Fig. 7). The approach was similar to one previously used to identify differences in deduced σ1 and σ3 amino acid sequences among reovirus isolates that show differences in biological phenotypes attributable to the σ1 and σ3 proteins (Dermody *et al.,* 1990; Kedl *et al.,* 1995; Chappell *et al.,* 1998). For this study, we focused our attention on the nine nucleotide positions that specify differences in the deduced λ1 sequences of T1L and T3D, rather than on sequencing the entire L3 genes of the additional isolates. Like T2J, the T3c10 and T3c18 L3 sequences (summarized in Table 3) revealed amino acids identical to T3D at four of the nine positions (alanine

TABLE 3

Deduced λ1 Amino Acid Sequences for Reovirus Isolates T2J, T3c10, T3c18, and T3c87 at the Nine Positions that Differ Between T1L and T3D λ1

Virus strain	Deduced amino acid at position no. in λ1									ATPase activity
	23	54	71	88	107	215	500	1011	1100	
T3D[a]	A[b]	K	M	K	K	D	I	I	L	T3D[c]
T1L[a]	S	R	L	R	Q	E	S	V	M	T1L
T2J[d]	A	K	L	K	K	E	S	V	M	T1L
T3c10[d]	A	K	L	K	K	E	S	V	M	T1L
T3c18[d]	A	K	L	K	K	E	S	V	M	T1L
T3c87[d]	A	K	T	K	K	E	S	V	M	T1L

[a] Nucleotide sequences were determined as described for Fig. 3.
[b] Single-letter code for amino acids. Amino acids unique to T3D among these isolates are underlined.
[c] Refer to Fig. 7 for assignment of ATPase activity as being like either T3D or T1L.
[d] Nucleotide sequences were determined from both strands of noncloned PCR products.

23, lysine 54, lysine 88, and lysine 107) and identical to T1L at the remaining five positions (leucine 71, glutamate 215, serine 500, valine 1011, and methionine 1100). Because T3c10, T3c18, and T1L exhibit similar ATPase phenotypes (Fig. 7), the simplest interpretation is, again, that one or more of the amino acids they share at positions 71, 215, 500, 1011, and 1100 cause their L3-determined ATPase phenotypes to be distinct from those of T3D. The T3c87 L3 sequence (also summarized in Table 3) was similar to those of T3c10 and T3c18 but exhibited a unique amino acid at position 71 (threonine). Because T3c87 and T1L exhibit similar ATPase phenotypes, this finding may suggest that the residue at position 71 has little influence on ATPase phenotypes. However, it is also possible that a methionine at position 71 is specifically required to generate the distinct ATPase phenotypes of T3D.

The sequence comparisons in Table 3 provide useful information for future studies involving site-directed mutagenesis of λ1 to assess its ATPase behavior (Bisaillon *et al.,* 1997). Because the putative helicase motifs are restricted to the N-terminal 440 residues of λ1 (Noble and Nibert, 1997a; Bisaillon *et al.,* 1997), the sequence polymorphisms at positions 71, 215, and 500 among the isolates tested in this study appear to be the best candidates for explaining their L3-determined differences in ATP hydrolysis. The partial L3 sequences of T3c10, T3c18, and T3c87 additionally demonstrated the same five insertions relative to the previously reported T3D L3 sequence (Bartlett and Joklik, 1988) as found in our T1L, T2J, and T3D L3 sequences. Last, the deduced sequences for T3c10, T3c18, and T3c87 λ1 were very similar to those of T1L and T3D in all of the sequenced regions (data not shown) and revealed conservation of both the six putative helicase motifs (Noble and Nibert, 1997a; Bisaillon *et al.,* 1997) and the putative zinc-binding residues (Bartlett and Joklik, 1988; Schiff *et al.,* 1988; Dermody *et al.,* 1991 Lemay and Danis, 1994) (data not shown).

MATERIALS AND METHODS

Cloning T1L and T3D L3 genes

Core-generated transcripts of reoviruses T1L and T3D were used for the synthesis of full-length cDNA copies of each L3 gene using a primer corresponding to the 18 5′-terminal nucleotides of minus strand from the T3D L3 sequence of Bartlett and Joklik (1988). This primer also included an *Eco*RI site at its 5′ end for use in cloning. After cDNA synthesis using AMV reverse transcriptase (GIBCO BRL) and removal of RNA, the original primer and a second corresponding to the 18 5′-terminal nucleotides of plus strand from the T3D L3 gene were used in performing amplification via PCR. The second primer also included an *Eco*RI site at its 5′ terminus. A mixture of *Taq* and *Pwo* polymerases (Expand Long Template; Boehringer-Mannheim) was used for PCR to reduce the frequency of mutations (Barnes, 1994). In addition to 2 min to warm to 92°C at the outset and 7 min at 68°C to elongate any partial products at the end, PCR cycles involved denaturation at 92°C for 30 s, annealing at 58°C for 35 s, and elongation at 68°C for 150 s for the first 10 cycles and for 170 s for the remaining 24 cycles. After PCR, the products were gel-purified, digested with *Eco*RI, and ligated into the *Eco*RI site of pBluescript KS(+) (Stratagene). Clones containing full-length copies of L3 were identified, and one clone each representing the T1L and T3D L3 genes was selected for further studies.

DNA sequencing of full-length clones and PCR products of L3 genes

DNA sequencing was performed at the University of Wisconsin Biotechnology Center DNA Sequencing Facility using their standard protocols for cycle sequencing with fluorescent dideoxy terminators (Applied Biosystems). The primers used for sequencing reactions with purified pBluescript-L3 plasmids were chosen to provide complete sequences from both strands of L3 in each clone. We also determined sequences from noncloned PCR products of the T1L and T3D L3 genes to address whether any nucleotides in the sequences of the cloned products might represent PCR-related mutations. With the noncloned PCR products of T1L and T3D L3, sequences were obtained from both strands only when discrepancies with the sequences of the clones were noted. In addition, the DNA products used for sequencing from opposite strands in these cases were derived from independent PCRs and demonstrated that the discrepancies did not result from PCR-related mutations in the noncloned products themselves. Only two nucleotides in the T1L L3 clone (980 and 1633) and two nucleotides in the T3D L3 clone (2082 and 2951) were found to differ from those in the noncloned PCR products. The sequences reported for L3 and λ1 in this report are those obtained from the noncloned products. Sequences were obtained only from noncloned PCR products for the L3 genes of isolates T2J, T3c10, T3c18, and T3c87. For T2J, sequences were obtained along the full lengths of both strands of the PCR products. In addition, the DNA products from independent PCRs were used for sequencing from regions on opposite strands of the T2J L3 and showed no evidence for PCR-related mutations in the sequences of the noncloned products. Sequences for residues 28–668 at the 5′ end of the T2J L3 coding strand (encompassing the hypervariable region in the deduced λ1 sequence) were additionally confirmed using a second, independent preparation of core-derived transcripts for reverse transcription, PCR amplification, and cycle sequencing. For T3c10, T3c18, and T3c87, primers were chosen to provide sequences from both strands of L3 across the regions in which differences were noted in

the deduced λ1 sequences of T1L and T3D. A mixture of *Taq* and *Pwo* polymerases (Expand Long Template) was used for generating all noncloned PCR products in an effort to reduce the frequency of mutations (Barnes, 1994). In addition to 2 min to warm to 92°C at the outset and 7 min at 68°C to elongate any partial products at the end, PCR cycles involved denaturation at 92°C for 30 s, annealing at 58°C for 35 s, and elongation at 68°C for 150 s for the first 10 cycles and for 170 s for the remaining 15 cycles.

Amplification of the L3 gene ends for DNA sequencing

To determine sequences from the ends of the T1L, T2J, and T3D L3 genes, we used modifications of protocols for 5′ RACE and 3′ RACE as described by Frohman *et al.* (1988). To determine 5′ sequences, full-length L3 cDNA was purified with a Qiaquick PCR purification kit (Qiagen). The 3′ end of the cDNA was then tailed with deoxyadenosine using terminal deoxynucleotidyl-transferase (New England Biolabs) according to the manufactor's instructions but with shortening of the reaction time to 10 min, followed by purification with Qiaquick. Last, the L3 segment 5′ end was amplified by PCR with Vent polymerase (New England Biolabs), terminal primer 5′-$(dT)_{19}$GC-3′, and an internal primer matched in melting temperature to the terminal primer. To determine the 3′ sequences, reovirus transcripts were tailed with poly(A)$^+$ polymerase (GIBCO BRL), according to Tabor (1998) except for shortening the reaction to 10 min, followed by phenol/chloroform extraction and ethanol precipitation. Synthesis of cDNA was then performed using reverse transcriptase (GIBCO BRL) and a $(dT)_{18}$ primer. Last, the 3′ end of the L3 segment was amplified by PCR with Vent polymerase (New England Biolabs), terminal primer 5′-$(dT)_{17}$GATG-3′, and an internal primer matched in melting temperature to the terminal primer. In addition to 2 min to warm to 92°C at the outset and 2 min at 72°C to elongate any partial products at the end, the first three PCR cycles involved denaturation at 92°C for 30 s, annealing at 46°C for 30 s, and elongation at 72°C for 39 or 52 s (depending on the length of the expected product) whereas the remaining 30 cycles involved denaturation at 92°C for 30 s, annealing at 46–31°C for 30 s (temperature decreased a half degree in each successive cycle), and elongation at 72°C for 39 or 52 s (again depending on the length of the expected product).

Frameshift mutant for premature termination of λ1

Nucleotide 3632 in the T3D L3 gene was deleted using the QuikChange site-directed mutagenesis protocol (Stratagene). Briefly, two complementary 45-nucleotide primers (5′-CCAATATATCATTTCAACTGAATATAATG-ATGGTCTCTGTTCTGC-3′ and 5′-GCAGAACAGAGACCA-TCATTATATTCAGTTGAAATGATATATTGG-3′) were used at a final concentration of 1 pmol/μl each. A pBluescript KS(+) plasmid into which T3D L3 nucleotides 2865–3901 had been subcloned was used as template for *Pfu* polymerase in a PCR as recommended by the protocol. After PCR, the wild-type template (containing methylation sites) was removed by successive treatments with *Dpn*I. The DNA was then transformed into *Escherichia coli* DH5α for amplification. The deletion of L3 nucleotide 3632 in the mutagenized plasmid was confirmed by sequencing, and the *Mlu*I–*Hin*dIII fragment of that plasmid was excised and used to replace the same fragment of the original full-length T3D L3 gene in pBluescript-L3-T3D. The presence of the deletion in the final plasmid was also confirmed by sequencing.

In vitro transcription and translation

The method for *in vitro* transcription was modified from that of Gurevich *et al.* (1991). Briefly, 2 μg of linearized pBluescript-L3 DNA was used in a 20-μl reaction containing 3 mM concentration each of ATP, CTP, and UTP; 1 mM GTP; 5 mM 7Me-GpppG cap analog (New England Biolabs); 50 mM HEPES (pH 7.5); 12 mM $MgCl_2$; 2 mM spermidine; 40 mM DTT; 200 U of T7 RNA polymerase (Epicentre), 20 U of RNasin (Promega); and 0.1 U of inorganic pyrophosphatase (Sigma). After incubation at 37°C for 90 min, the same amounts of enzymes and RNasin were added, and the reaction was incubated for an additional 90 min at 37°C. The L3 RNA was precipitated by adding an equal volume of 5 M ammonium acetate, incubation on ice for 15 min, and centrifugation at 16,000*g* for 15 min at 4°C. The pellet was washed with cold 70% ethanol and resuspended in 20 μl of H_2O. The Red Nova Lysate system (Novagen) was then used for *in vitro* translation according to the instructions for capped RNAs. The translation reaction was incubated at 30°C for 90 min.

Protein electrophoresis

Electrophoresis using both reduced-bis Tris–glycine and phosphate–urea SDS gels was performed as described by Dryden *et al.* (1998).

ATPase assays

ATPase assays were performed as described by Noble and Nibert (1997a).

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